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IMMUNOTOXICOLOGICAL INVESTIGATIONS IN THE MOUSE: GENERAL APPROACH AND METHODS

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ABSTRACT

The adverse effects of chemicals on the lymphoreticular system have generated considerable toxicological interest. In this series of papers, the effects of selected environmentally relevant compounds are reported. This first paper describes the methods and general approach used in judging a chemical's potential risk to the immune system. Risk evaluation was approached utilizing acute, 14- and 90-day studies. Both sexes of the CD-1 random-bred mouse were employed. The immune system was evaluated against a background of more standard toxicological parameters, which included fluid consumption, body and organ weights, hematology, serum and liver chemistries, hepatic microsomal enzyme activities and blood coagulation. Bone marrow status was evaluated by assessing DNA synthesis. Humoral immunity was evaluated by determining the number of IgM spleen antibody-forming cells (AFC) to sheep erythrocytes (sRBC), the serum antibody level to sRBC, and spleen lymphocyte response to the B cell mitogen, lipopolysaccharide (LPS). The status of cell-mediated immunity was assessed by quantitating the delayed type hypersensitivity (DTH) response to sRBC, proliferation of the popliteal lymph node, and the spleen cell response to the T lymphocyte mitogen, Concanavalin A (Con A). Macrophage function was evaluated by measurement of the vascular clearance rate and distribution of radiolabeled sRBC in the liver, spleen, lungs, and thymus, and

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recruitability, adherence, chemotaxis, and phagocytic activity of peritoneal exudate cells (PEC). Historical control data from six 14- and 90-day studies conducted over a one year period are given. The data resulting from these types of studies can provide a basis for the initial evaluation of a chemical's adverse effect on the immune system.

INTRODUCTION

The adverse effects of chemicals on the organs, tissues, and cells of the lymphoreticular system have received considerable attention from both toxicologists and immunologists. This interest is well founded since the immune system has been shown to be the target organ of certain chemically and physically diverse compounds[1,2,3]. This system, like all other systems in the body, is complex, with several types of cells working both independently and in concert, to carry out a role in homeostasis. Immunotoxicology is a subject gaining increased activity and awareness because the physiology and biochemistry of the immune system are now being systematically dissected; as a result, the basic processes involved in host defense mechanisms are better understood. A second reason for the interest in immunotoxicology is that it represents a system in which the cells from exposed animals can be readily removed and their function(s) examined *in vitro*. This is in keeping with the direction that toxicology is taking, i.e., complementing morphological changes with functional alterations.

This paper describes the approach we have taken in evaluating the immunotoxicity of chemicals in experimental random-bred mice. The effects of a given chemical on the immune system were investigated against a background of standard toxicological procedures. In this way, the specificity of the immune system as a target could be more clearly defined. Historical control data are provided for each of the assays, with the data presented as the mean ± SD. In addition, an analysis of variance was calcu-

lated on the combined experiments to determine if there were significant differences among the control values from experiment to experiment.

EXPERIMENTAL APPROACH

Figure 1 outlines the experimental design which is now being used to evaluate chemicals for their target organ toxicity.

After the compound was selected for study, unknown physical and chemical properties of the chemical were determined and necessary studies were performed to assure appropriate integration into the *in vivo* systems. These data included: confirmation of identity, solubility properties, pH characteristics, stability at concentrations in the test system, and purity.

Acute toxicity studies were performed on eight week old male and female mice using the route of administration which most closely approximates human exposure. Routinely, eight mice were used per group and at least six dose levels were employed. In many of these studies, we were concerned with environmental chemicals that are introduced orally. The mice were exposed via an 18 gauge stainless steel stomach tube after 18 hr of fasting. The mice were observed hourly for the first eight hours for behavioral changes and morbidity, then twice daily thereafter for 14 days. The animals still alive at the end of the experimental period were sacrificed, and all mice were necropsied and examined for gross pathology. Calculations of the LD_{50} and slopes of the dose-response curves with 95% confidence limits were performed by the Probit Procedure of Goodnight[4]. These data provided the basis for selecting the dose levels employed in the 14- and 90-day studies, and began to indicate the possible target organ(s).

Following the acute studies, a 14-day range finding study was performed. If no sex differences were seen in the acute toxicity, only male mice were used. Again, the relevant route

FLOW CHART FOR IMMUNOTOXICOLOGICAL STUDIES

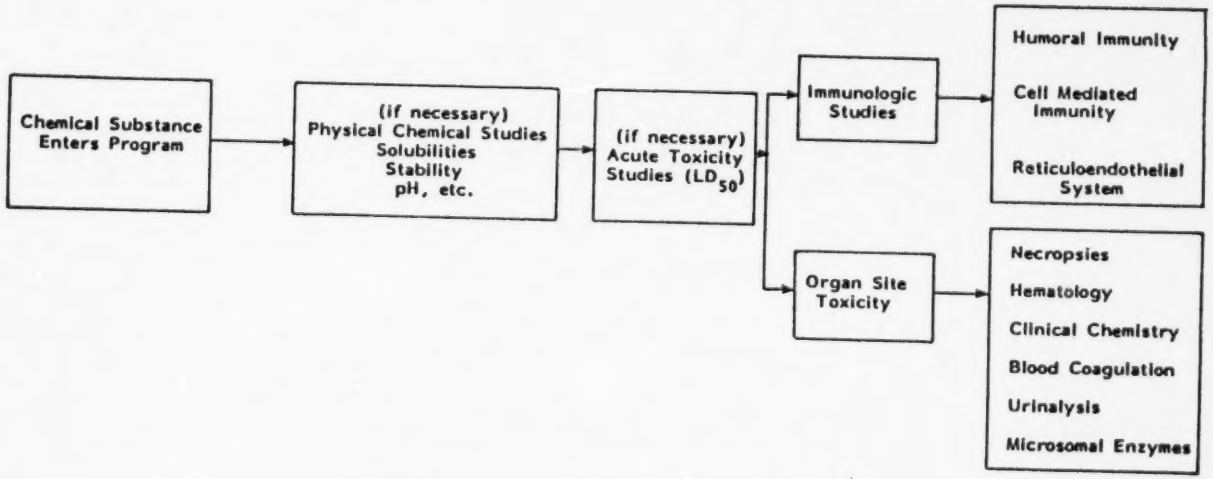

FIG. 1. Flow chart for immunotoxicological studies.

of exposure was employed. The chemical was administered daily for 14 days via stomach tube at two dose levels. These levels were usually 1/10 and 1/100 the LD_{50}. Body weights were determined and recorded prior to the initiation of exposure, and again one and two weeks after exposure.

Table 1 lists the variables monitored after 14 days of exposure. Three sets of mice were used to obtain this toxicological information. In one set of mice, humoral immunity was assessed by enumerating the number of spleen IgM AFC to sRBC, both as a function of AFC per spleen and per 10^6 spleen cells. In the same mice, serum obtained from blood collected by cardiac puncture was used for serum chemistry studies. These included glutamic-pyruvic transaminase, lactate dehydrogenase and blood urea nitrogen. A second set of mice was used for general toxicological assessment. Cardiac blood was collected into 3.2% sodium citrate (1:10 citrate to blood) for hematology and coagulation studies. Hematological studies included leukocyte counts, hematocrit, and hemoglobin levels. If a change was seen in leukocyte counts, a differential analysis was performed. A complete necropsy was performed on this set of mice and the following organs were removed, trimmed, and weighed: brain, liver, spleen, lungs, thymus, kidneys, and testes. The organ weight data were expressed as both organ to body weight ratio and as organ to brain weight ratio. Cell-mediated immunity was assessed in the third set of mice by measurement of the DTH response to sRBC.

Tables 2-6 show the control values for CD-1 mice in the 14-day studies. These values were derived from six 14-day studies performed over a one-year period. The mice were five weeks of age when they arrived at our facility. They were quarantined for seven days and then placed in the study for the two-week period. The 14-day study provided baseline data for the design of the 90-day study. Except for mutagenic, carcinogenic, teratogenic, and reproductive effects, the 90-day study should successfully describe the target organ toxicity.

TABLE 1
Parameters Measured in a 14-Day Study[a]

Standard Toxicology

1. Body Weights
2. Necropsy - Gross Pathology
3. Organ Weights
4. Hematology
5. Serum Chemistries
6. Blood Coagulation

Immunotoxicology

1. Humoral immunity - spleen AFC response to sRBC
2. Cell-mediated immunity - DTH response to sRBC

[a]Doses were based on the acute toxicity study and were usually 1/10 and 1/100 of LD50.

TABLE 2
Control Values for Body Weights (Grams) of Two Month Old Male CD-1 Mice[a]

Final Weight	Initial Weight	Change in Weight
30.0 ± 2.8	24.2 ± 2.3	5.8 ± 2.3

[a]Values represent the mean ± SD of 334 control male CD-1 mice derived from six 14-day experiments. All weight means were significantly different ($p < 0.05$) among the six experiments.

Table 7 lists the variables that were measured during, and at the conclusion of, the 90-day study. Six sets of mice were used to evaluate these variables. The first set was used for necropsy (gross pathology), organ weights, microsomal mixed functional oxidase parameters, liver glutathione levels, hematology, coagulation, hemagglutination titer to sRBC and bone marrow DNA synthesis. The second set of mice was used to

TABLE 3
Control Organ Weight Values of Two Month Old Male CD-1 Male[a]

Organ	Weight (mg)	% Body Weight	Organ/Brain
Brain	440 ± 39	1.47 ± 0.15*	-
Liver	1862 ± 294*	6.17 ± 0.73	4.26 ± 0.74
Spleen	170 ± 53	0.56 ± 0.17	0.39 ± 0.12
Lungs	214 ± 35	0.71 ± 0.11	0.48 ± 0.07
Thymus	79 ± 19	0.26 ± 0.06	0.18 ± 0.04*
Kidney	522 ± 84	1.73 ± 0.20	1.19 ± 0.15
Testes	211 ± 34	0.70 ± 0.11	0.48 ± 0.07*

[a]Values represent the mean ± SD of 68-70 control male CD-1 mice derived from six 14-day experiments. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

TABLE 4
Control Values for Selected Hematological, Serum Chemistry and Blood Coagulation Parameters in Two Month Old Male CD-1 Mice[a]

Parameter	Values
Hemoglobin (g%)	12.7 ± 2.1
Hematocrit (%)	41.2 ± 2.5
Leukocytes ($10^3/mm^3$)	7.44 ± 2.76
Lactate Dehydrogenase (IU/L)	926 ± 195
Glutamic-Pyruvic Transaminase (IU/L)	56.4 ± 21.6
Blood Urea Nitrogen (mg%)	27.2 ± 4.8
Prothrombin Time (sec)	8.4 ± 0.8
Fibrinogen (mg%)	298 ± 41

[a]Values represent the mean ± SD derived from 68 control mice used in six 14-day studies. All values were significantly different ($p < 0.05$) among the six experiments.

TABLE 5
Control Values for Humoral Immune Assessment in Two Month Old Male CD-1 Mice[a]

Parameter	Values
Spleen Weight (mg)	204 ± 47
Spleen Cell Number ($\times 10^{-8}$)	1.65 ± 0.48
IgM AFC/Spleen ($\times 10^{-5}$)	4.86 ± 3.18
IgM AFC/10^6 Spleen Cells	2904 ± 1667

[a]Values represent the mean ± SD derived from 68 control mice in six 14-day studies. The antibody forming cells were enumerated on the peak day of response (day 4). All parameters were significantly different ($p < 0.05$) among the six experiments.

TABLE 6
Control Values for the DTH Response to sRBC in Two Month Old Male CD-1 Mice[a]

Parameter	Value
Stimulation Index	3.73 ± 1.60

The value represents the mean ± SD derived from 70 control mice used in six 14-day studies. A value of 1.97 has been subtracted from each animal value to correct for non-specific swelling as described in Methods. There was no significant difference ($p < 0.05$) among the six experiments.

determine spleen AFC response to sRBC, spleen cell response to mitogens, and serum chemistries. Assessment of cell-mediated immunity, as measured by the DTH response to sRBC, was accomplished with the third set of mice, while the fourth set of mice was used to determine popliteal lymph node proliferation. The fifth set of mice was used to measure the functional activity of

TABLE 7
Parameters Measured in a 90-Day Study

A. Standard Toxicology

1. Body weights - twice weekly
2. Fluid consumption
3. Necropsy - gross pathology
4. Organ weights - brain, liver, spleen, lungs, thymus, kidneys, and testes
5. Hematology - hematocrit, erythrocytes, leukocytes, differential, platelets, and hemoglobin
6. Coagulation - prothrombin time, activated partial thromboplastin time, and fibrinogen
7. Serum chemistries - lactate dehydrogenase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, alkaline phosphatase, blood urea nitrogen, total protein, glucose, cholesterol, creatinine, phosphorus, calcium, sodium, chloride, and potassium
8. Liver chemistries - glutathione levels

B. Hepatic Microsomal Mixed Functional Oxidase Parameters

1. Liver weight
2. Microsomal protein
3. P450 content
4. Cytochrome b_5
5. Aminopyrine-N-demethylase
6. Aniline hydroxylase

C. Cell Mediated Immunity

1. DTH response to sRBC
2. Popliteal lymph node proliferation to sRBC
3. Spleen cell response to Con A

D. Humoral Immunity

1. Spleen AFC response to sRBC
2. Serum antibody response to sRBC
3. Spleen cell response to LPS

E. Functional Activity of the Reticuloendothelial System

1. Vascular clearance rate of sRBC
2. Organ uptake of sRBC
3. Chemotaxis, adherence, and phagocytic activity of PEC

F. Bone Marrow

1. DNA synthesis

the reticuloendothelial system, while the sixth set of mice was used to study the number and functional status of PEC. Tables 8-18 show the control values for CD-1 mice in the 90-day studies.

METHODS

Experimental Animals

Although the mouse is currently being used for some toxicological studies, the random bred rat has been the experimental animal most widely used and considered to be the most appropriate for toxicological evaluation of chemicals and drugs. Unfortunately, the physiology of the rat's immune system has not been studied or defined to the same degree as that of the mouse and man. The mouse has been the immunologist's animal of choice because of the availability of inbred strains, which has allowed for the development of specific immune sera and for decreased animal-to-animal variation. In addition, due to the homogeneity of the lymphoid cells derived from genetically similar inbred mice, functional tests can be performed on cell samples pooled from several animals.

As a compromise, the program we have developed employs the random bred CD-1 mouse. Of the three commercial sources of mice we evaluated, only one (Charles River Breeding Laboratories, Wilmington, MA) supplied mice of consistent quality and temperament. This mouse has been used to assess the effects of chemicals on the immune systm, and with the increased availability of microassays, has become attractive for the more routine toxicological tests.

Upon arrival, all animals were housed four per cage in plastic shoe box cages containing sawdust bedding (PWI Hardwood Sawdust, Lowville, NY). After the quarantine period (7 days), animals were randomized and were individually earpunched. Acute toxicity studies were performed on animals eight weeks of age.

TABLE 8
Control Values for Body Weight (Grams) of Male and Female CD-1 Mice Used in 90-Day Studies[a]

	Final Weight	Initial Weight	Change in Weight
Males	39.3 ± 4.6	22.2 ± 3.6	17.3 ± 5.7
Females	30.6 ± 3.8	19.5 ± 2.8	11.1 ± 4.5

[a]Values represent the mean ± SD of 288 male and 288 female control mice derived from six 90-day studies. All weights were significantly different ($p < 0.05$) among the six experiments.

TABLE 9
Control Hematology Values for Male and Female CD-1 Mice in 90-Day Studies[a]

Parameter	Male	Female
Hemoglobin (g%)	12.5 ± 2.0	12.3 ± 2.0
Hematocrit (%)	41.3 ± 3.7	41.4 ± 2.0*
Erythrocytes ($10^6/mm^3$)	8.19 ± 1.59	8.51 ± 2.05
Leukocytes ($10^3/mm^3$)	6.42 ± 2.79	6.37 ± 2.94
Platelets ($10^5/mm^3$)	3.78 ± 0.90	3.60 ± 0.81
Protnrombin time (sec)	9.6 ± 0.7	9.7 ± 0.7
APTT	31.1 ± 5.5*	33.1 ± 5.8*
Fibrinogen (mg%)	282 ± 50	211 ± 29
Differential:		
Lymphocytes (%)	67.2 ± 15.8	78.7 ± 9.0
Polymorphonuclears (%)	30.0 ± 15.4	16.1 ± 7.9
Monocytes (%)	3.8 ± 3.3	3.6 ± 2.8
Eosinophils (%)	1.0 ± 1.3*	1.6 ± 1.2*

[a]Hematology values represent the mean ± SD of 131 male and 137 female control CD-1 mice derived from six 90-day studies. Differential values represent the mean ± SD of 88-92 male and female CD-1 mice derived from four subchronic 90-day studies. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

The 14-day studies were begun on animals six weeks of age, while four week old animals were used in 90-day studies. Mice exposed by gavage were maintained on Purina Lab Chow and water ad libitum. Those animals exposed to the chemicals in drinking water were maintained on Purina Lab Chow and chemical solutions ad libitum. Control values for body weights of the mice used in the 90-day studies are presented in Table 8. Animal room temperature was maintained between 21 and 24°C and relative humidity between 40-60%. The light-dark cycle was maintained on 12-hr intervals.

If the chemical was water soluble it was placed directly in deionized water. For the chemicals which were water insoluble, a polyethoxylated vegatable oil, emulphor (GAF 620, GAF Corp., New York, NY), was used as the vehicle. Ten percent emulphor in deionized water was used to dissolve chemicals used in gavage studies, while a 1% emulphor solution was used for drinking water studies, if necessary. Solutions for gavaging were prepared fresh daily, while solutions for the drinking water were prepared twice weekly. All solutions were maintained in dark bottles to alleviate possible photodecomposition. Gavaging was done with an 18 gauge stainless steel feeding tube between the hours of 10:00 and 11:00 a.m. Solutions for drinking water studies were placed in 250-500 ml amber colored bottles capped with cork stoppers containing stainless steel sipper tube inserts. All drinking water solutions were analyzed by GLC with head space analysis to determine the stability of the compound over a three- to four-day period at room temperature. Fluid consumption was calculated by weighing the water bottles at the time solutions were initially placed in them, then weighing the bottles again before solutions were changed to determine the amount consumed over the three- or four-day period.

Blood and Tissue Collection, Hematology, and Coagulation Studies

To assure that the maximum amount of blood was obtained and that its quality was applicable for special coagulation tests,

the mice were anesthetized with chloroform. Several anesthetic agents have been tried, and the two that did not perturb any of the parameters examined, including the hepatic microsomal mixed functional oxidase system, were chloroform and sodium brevital. Sodium pentobarbital and hexobarbital perturbed liver microsomal enzymes and ether affected the quality and amount of blood drawn from the animals (data not shown). The blood was collected by cardiac puncture into a 3 ml plastic syringe fitted with a 23 gauge needle. For the hematology and blood coagulation studies, the blood was collected into 3.2% sodium citrate (1:10 citrate to blood). The samples were collected without air bubbles within 30 sec of the puncture. One to two ml of blood were routinely collected by this method. The blood was maintained at 4°C and leukocytes, erythrocytes, and platelets were enumerated on a Coulter counter, Model ZBI. Platelet counts were determined on platelet-rich plasma by a modification of the methods of Bull et al.[5] Leukocyte differentials were evaluated using the standard Wright's-Giemsa staining procedure. Hematocrits were performed on a micro-hematocrit centrifuge and hemoglobins determined by the cyanomethemoglobin method. The plasma was separated from the cellular elements by 800 xg centrifugation at 4°C. The plasma was used to evaluate the status of the coagulation system. Prothrombin times were performed to assess extrinsic activity and activated partial thromboplastin times (APTT) were performed to assess intrinsic activity. These assays were done on a BBL Fibrometer using General Diagnostics reagents (Baltimore, MD). Fibrinogen levels were determined by the kinetic method using Dade Diagnostics reagents (Miami, FL). Control historical hematologic and coagulation data from six 90-day studies are shown in Table 9. The only parameter showing a sex difference was the fibrinogen level, where the females had a 25% lower level than the males.

Immediately after the blood sample was drawn, the liver was examined, removed, trimmed, weighed, and if needed, a section was removed for histopathological examination. The remaining liver tissue was homogenized and a microsomal fraction was prepared for analysis. One femur was removed and the bone marrow collected for analysis. A complete necropsy was then performed and the following organs were removed, trimmed, and weighed: brain, liver, spleen, lungs, thymus, kidneys, and testes. Samples of these tissues were fixed in 10% buffered formalin along with the heart, mesenteric lymph nodes, striated muscle, stomach, ileum, jejunum, colon, bladder, ovaries, and adrenals. The most notable sex differences in organs weights were in the brain and kidney (Table 10). The brain was 28% larger in the female when calculated on a percent body weight basis. The kidney was 17% and 35% larger in the male when calculated on a percent of body weight and organ to brain ratio, respectively.

Preparation of Microsomes

Immediately following sacrifice, livers were removed, weighed, thoroughly rinsed, and homogenized in 9 volumes of ice cold 0.15M KCl - 0.01M potassium phosphate buffer (pH 7.4) using a teflon glass homogenizer, and centrifuged at 9000 xg for 20 min. An aliquot of the supernatant fluid was removed, carefully avoiding the top lipid layer, and recentrifuged at 105,000 xg for 60 min. The resulting pellet was rinsed and resuspended in 0.05M potassium phosphate buffer to obtain a concentration of approximately 2 mg protein per mg of buffer. The protein concentration of the microsomal suspension was determined by the method of Lowry et al.[6], using human serum albumin as the standard.

Drug Metabolism Assays

Substrates were selected as representative of Type I (aminopyrine) and Type II (aniline) cytochrome P450-dependent mixed

TABLE 10
Control Organ Weight Values for CD-1 Mice
Used in 90-Day Studies[a]

Organ	Weight (mg)	% Body Weight	Organ/Brain
		MALES	
Brain	499 ± 39	1.27 ± 0.14	
Liver	2057 ± 349	5.17 ± 0.53	4.13 ± 0.70
Spleen	167 ± 69	0.42 ± 0.16	0.34 ± 0.14
Lungs	234 ± 36	0.59 ± 0.08*	0.47 ± 0.07
Thymus	45 ± 14	0.11 ± 0.03	0.09 ± 0.03
Kidney	650 ± 102	1.64 ± 0.18*	1.30 ± 0.20
Testes	260 ± 41	0.66 ± 0.10*	0.52 ± 0.08*
		FEMALES	
Brain	497 ± 41	1.63 ± 0.22	-
Liver	1526 ± 242	4.97 ± 0.68	3.10 ± 0.59
Spleen	155 ± 45	0.50 ± 0.13	0.32 ± 0.10
Lungs	216 ± 40	0.71 ± 0.13	0.44 ± 0.09
Thymus	54 ± 16	0.17 ± 0.05	0.11 ± 0.03
Kidney	418 ± 54	1.36 ± 0.16	0.85 ± 0.13

[a]Values represent the mean ± SD of 132 male and 138 female control CD-1 mice derived from six 90-day studies. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

function oxidase. Historical control data for drug metabolism assays are shown in Table 11.

Aminopyrine N-demethylase: The methods of Cochin and Axelrod[7] were used to measure the in vitro metabolism of aminopyrine. A 25 ml Ehrlenmeyer flask containing 0.5 ml cofactors and buffer (7.5 µmol $MgCl_2 \cdot 6H_2O$, 17 µmol glucose-6-phos-

TABLE 11
Control Values for Hepatic Microsomal Mixed Functional Oxidase Parameters of Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Unit	Male	Female
Microsomal Protein	mg/g liver	23.4 ± 3.2	20.4 ± 2.3
Cytochrome P450	nmol/mg protein	1.16 ± 0.259	0.98 ± 0.184
Cytochrome b5	nmol/mg protein	0.41 ± 0.050	0.52 ± 0.079
Aminopyrine N-Demethylase	nmol/mg protein/min	10.6 ± 1.7	13.1 ± 2.1
Aniline Hydroxylase	nmol/mg prot/min	1.60 ± 0.48	1.72 ± 0.37

[a]Values represent the mean ± SD of 44 male and 48 female mice from six 90-day studies. The means of the parameters were significantly different ($p < 0.05$) among the experiments.

phate, 2 µmol NADP, and 1 unit glucose-6-phosphate dehydrogenase in 0.5M potassium phosphate buffer, pH 7.4), 0.5 ml aminopyrine (10 µmol), and 1.0 ml of microsomal suspension (2 mg protein/ml) was prepared. Blanks were prepared similarly, except that aminopyrine was omitted from the flasks. The flasks were shaken for 10 min at 37°C at 120 oscillations per min in a gyratory shaker bath. At the end of the incubation period, the reaction was terminated by the addition of 0.6N perchloric acid. The contents were transferred to centrifuge tubes and centrifuged at 2000 xg for 15 min. One ml of the supernatant fluid was added to 1 ml of double strength Nash reagent (300 g ammonium acetate and 2 ml acetyl acetone per liter H_2O), and the color allowed to develop for 30 min in a 60°C water bath. Samples were cooled immediately and the absorbance at 415 nm was recorded.

Aniline Hydroxylation: The 4-hydroxylation of aniline was determined using a comparable procedure[8], except that 16 µmol of aniline HCl was incubated in the presence of cofactor and

microsomes. The reaction was terminated by addition of 1.0 ml of 20% trichloroacetic acid. One ml of the protein-free supernatant fluid was added to 0.5 ml 10% sodium carbonate, mixed, and added to 1.0 ml of phenol reagent (2% phenol in 0.2N NaOH). The color was allowed to develop at 37°C for 30 min and the absorbance at 630 nm recorded.

Spectral Studies

The methods of Omura and Sato[9] were used to measure the content of microsomal cytochromes P450 and b_5. Historical control data are shown in Table 11.

Cytochrome P450: Microsomes were diluted to 1 mg protein per ml in 0.1M potassium phosphate buffer. Carbon monoxide was gently bubbled through the sample cuvette for 30 sec and a few crystals of sodium dithionite were added to each cuvette. The CO-difference spectrum of the reduced microsomes was recorded from 500-400 nm and cytochrome P450 content was calculated from the absorbance difference (OD 450 nm minus OD 490 nm) using 91 mM^{-1} cm^{-1} as the mM extinction coefficient.

Cytochrome b_5: Microsomes were diluted to 1 mg protein per ml buffer and divided equally into two cuvettes. The sample cuvette was reduced by adding 4.4 µmol NADH in 0.01 ml H_2O. The reduced vs oxidized spectrum was recorded and cytochrome b_5 content calculated from the absorbance difference (OD 423 nm minus OD 409 nm), using 185 mM^{-1} cm^{-1} as the mM extinction coefficient.

Bone Marrow

The status of the bone marrow was evaluated by determining bone marrow cell DNA synthesis. In addition, bone marrow smears were prepared for evaluation if marked changes in the parameter occurred. Bone marrow cells were added to each well of a 96-well microtiter dish at 6×10^5 cells/200 µl. Twenty µl of a solu-

tion containing 0.1 μCi of ^{125}I-Iododeoxyuridine (^{125}I-IUdR; New England Nuclear) in 2 x 10^{-5}M Fluorodeoxyuridine (FUdR; Sigma Chemical Co.) was added to each well. At 60 and 120 min, triplicate wells were harvested and the cells were collected onto filter disks with the aid of a Titertek cell harvester. Filter disks containing the ^{125}I-IUdR incorporated into DNA were radioassayed in a Beckman 300 gamma counter. Figure 2 shows a typical incorporation curve for ^{125}I-IUdR into bone marrow cells. As can be seen, there is a linear incorporation of ^{125}I-IUdR over a 3 hr incubation period. Appropriate studies were performed to assure that ^{125}I-IUdR was incorporated into DNA. Table 12 shows control historical data on bone marrow DNA synthesis.

Serum and Liver Chemistries

The serum enzymes, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, lactate dehydrogenase, and alkaline phosphatase were measured kinetically on the ABA 100 Bichromatic analyzer (Abbott Laboratories, Dallas, TX). These enzymes had been optimized for the mouse as to pH and substrate concentration. A-GENT reagent kits purchased from Abbott Laboratory Diagnostic Division (South Pasadena, CA) were used to determine blood urea nitrogen, total protein, glucose, cholesterol, and calcium, while Pierce reagent kits (Pierce Chemical Co., Rochford, IL) were used to measure phosphorus and creatinine. These chemistries were measured by endpoint analysis on the above-named instrument. Sodium and potassium ions were measured on a flame photometer (Instrumentation Laboratory 443), and chloride ions were measured on a chloridometer (Buchler Instruments). The methods of Jollow *et al.*[11] were used to measure liver glutathione content. A mixture of 0.5 ml of liver homogenate (described previously) and 0.5 ml of 4% sulfosalicylic acid was centrifuged at 2000 xg for 15 min. Two hundred μl of protein-

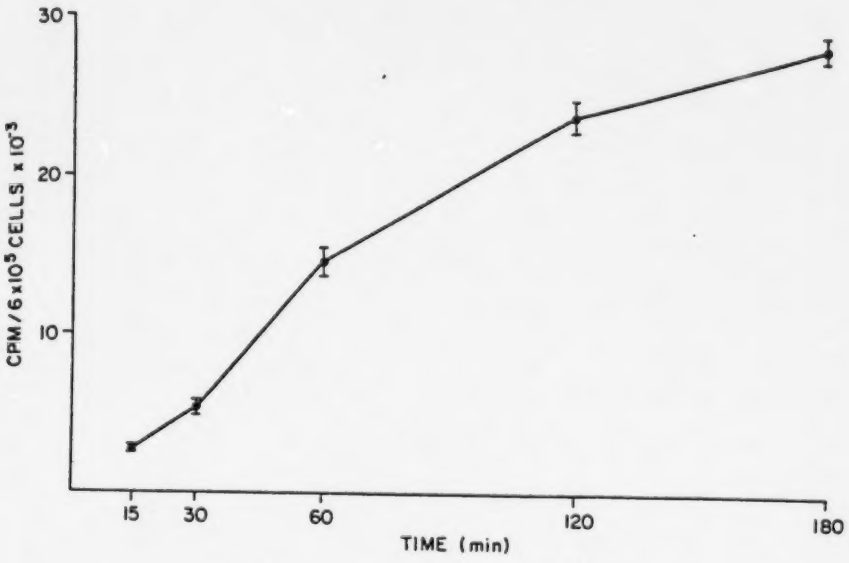

FIG. 2. The time course of incorporation of ^{125}I-IUdR into bone marrow cells over a 180 min period. See Methods for procedure.

TABLE 12
Control Values for Bone Marrow DNA Synthesis from Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Male	Female
DNA Synthesis (60 min)	16082 ± 4411	17246 ± 4319
DNA Synthesis (120 min)	29563 ± 6655	30224 ± 8823

[a]Values represent the mean ± SD of 60 male and female mice from six 90-day studies. There were no significant differences ($p < 0.05$) among the six experiments.

free supernatant fluid were added to 2.0 ml of 0.4 mM Ellman's Reagent (5,5'-dithiobis-2-nitrobenzoic acid) in 0.1M potassium phosphate buffer, pH 8.0. Ten min after mixing, the absorbance at 412 nm was recorded and compared to the absorbance of glutathione standards. Baseline historical control data for the serum and liver chemistries from the 90-day studies are shown in Table 13.

Cell-Mediated Immunity

The functional status of both the afferent and efferent arms of cellular immunity was evaluated by measuring a DTH response to sRBC. Sheep erythrocyte sensitization was done on Day 0 in the left hind footpad (LFP) with 1×10^8 sRBC in a volume of 20 µl. Four days following sensitization, the mice were challenged in the same footpad with 4×10^8 sRBC in a volume of 40 µl. Seventeen hr following the challenge, mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (^{125}I-HSA; 80,000 cpm/0.1 ml; Mallinckrodt). Two hr later, the mice were sacrificed by cervical dislocation and both hind feet were removed at the ankle joint and radioassayed in a gamma counter. It has been shown that ^{125}I-HSA will extravasate into the edematous area produced by a DTH response (Paranjpe and Boone[11]). The right hind footpad (RFP) served as a control for background infiltration of ^{125}I-HSA. A group of mice which were unsensitized but challenged as above acted as unsensitized controls to determine non-specific swelling. Results are expressed as a stimulation index (SI), which was calculated are follows:

$$SI = \frac{\text{LFP sensitized}}{\text{RFP sensitized}} - \text{mean } \frac{\text{LFP unsensitized.}}{\text{RFP unsensitized}}$$

Evaluation of cell-mediated immunity using the footpad assay requires both the afferent arm (antigen recognition and processing, blastogenesis) and the efferent arm (lymphokine production,

TABLE 13
Control Serum and Liver Chemistry Values in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Unit	Male	Female
LDH	IU/L	664 ± 233	606 ± 193
SGPT	IU/L	44.6 ± 24.4	36.5 ± 18.6
SGOT	IU/L	84.4 ± 38.9	92.0 ± 28.2
SAP	IU/L	44.1 ± 22.6	62.4 ± 29.7
Glutathione	µmol/g liver	8.97 ± 1.79	7.85 ± 1.66
BUN	mg%	27.7 ± 6.9	22.8 ± 5.2
Protein	g	7.13 ± 1.21	7.56 ± 1.08
Glucose	mg%	160 ± 32	137 ± 24
Cholesterol	mg%	167 ± 98	120 ± 77
Creatinine	mg%	0.42 ± 0.19	0.50 ± 0.23
Phosphorus	mEq/L	8.40 ± 2.01	8.05 ± 2.28
Calcium	mEq/L	12.0 ± 2.1	11.7 ± 1.6
Sodium	mEq/L	155 ± 10	156 ± 11
Chloride	mEq/L	108 ± 12	109 ± 14
Potassium	mEq/L	7.30 ± 0.94	6.90 ± 0.95

[a]Values represent the mean ± SD derived from 70-143 control CD-1 mice used in six 90-day studies. LDH=lactate dehydrogenase, SGPT=serum glutamic-pyruvic transaminase, SGOT=serum glutamic-oxaloacetic transaminase, SAP=serum alkaline phosphatase, BUN=blood urea nitrogen. The means of each parameter were significantly different ($p < 0.05$) among the six experiments.

increased vascular permeability) to function adequately. If an experimental chemical decreases the extravasation of ^{125}I-HSA, it may be evaluated as immunosuppressive in the cell-mediated response. However, this may not necessarily be true if the compound has anti-inflammatory properties. To determine if a chemical affects the afferent arm of the cellular response, prolifer-

ation of the popliteal lymph node cells to sRBC was measured. Additional groups of animals were used for this assay. They were subjected to the same procedure as were the DTH animals, except that 1 1/2 hr after challenge they received 20 µg/kg FUdR intraperitoneally, and 2 hr after challenge they received 1 µCi of ^{125}I-IUdR intravenously. ^{125}I-HSA was not administered to these animals. These mice were sacrificed 24 hr after challenge and both popliteal lymph nodes were removed and counted in a gamma counter. Non-specific proliferation was corrected for and an SI was calculated as described for the footpad assay. Historical control data for both footpad swelling and popliteal lymph node proliferation are shown in Table 14.

Lymphocyte Responsiveness

The mitogens employed were Con A, a T cell mitogen in the mouse, and LPS, a B cell mitogen. A 0.05 ml volume of RPMI 1640 medium containing either 1, 5, or 10 µg of Con A or 1, 5, or 20 µg of LPS was placed in flat-bottom microtiter wells and frozen at -70°C. When needed, plates were removed from the freezer and thawed at room temperature. No differences were observed between mitogen plates frozen and those freshly prepared. To detect a maximal mitogen response and to detect any shifts in the dose response curve, three concentrations of mitogen were routinely used.

Spleens were aseptically removed from mice and single cell suspensions prepared by pushing spleens through sterile 60-mesh wire screens into RPMI 1640 media supplemented with 5% heat-inactivated fetal calf serum (HIFCS), 2 mM L-glutamine, 100 µg/ml penicillin and 1 µg/ml streptomycin. The cell concentration was adjusted to 5 x 10^6 cells/ml in RPMI medium supplemented with 10% HIFCS, L-glutamine, and pencillin-streptomycin. One tenth ml of the cell suspension was placed in thawed microtiter wells containing mitogen; additionally, wells were set up without mitogen to act as background controls.

TABLE 14
Control Values for Cell-Mediated Immune Response as Measured by DTH and Popliteal Lymph Node Proliferation to sRBC in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Stimulation Index	Male	Female
Footpad Swelling	4.02 ± 2.05	4.61 ± 2.15
Popliteal Lymph Node Proliferation	18.0 ± 12.1	12.0 ± 6.7*

[a]Footpad swelling values represent the mean ± SD of 103 male and 104 female mice derived from six 90-day studies. A value of 1.95 for males and 2.07 for females has been subtracted from each animal to correct for non-specific swelling as described in Methods. Popliteal lymph node data were obtained from three 90-day studies using 29 males and 32 females. To correct for non-specific lymph node proliferation, a value of 1.1 for males and 1.3 for females was subtracted from each animal. The means of each parameter were significantly different ($p < 0.05$) among the experiments, except where indicated by an asterisk.

The plates were then placed in a 10% CO_2, 37°C humidified incubator. After 48 hr, 0.05 ml of a radiolabeled solution was added to each well. This solution contained ^{125}I-IUdR (4 μCi/ml) and FUdR (4×10^{-6}M). The plates were reincubated for 18-20 hr, at which time the cells were collected on filter disks using a Titertek cell harvester. The filter disks were then counted in a gamma counter. There are two ingredients which were essential for this assay to work: heat-inactivated fetal calf serum and FUdR.

Historical control data for lymphocyte responsiveness to Con A and LPS are shown in Table 15.

Humoral Immunity

Hemolytic Plaque Assay: The method used in our laboratory to detect AFC was the Cunningham modification of the Jerne

TABLE 15
Control Values for Spleen Lympnocyte Responsiveness to Con A and LPS in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Male	Female
	(cpm/5 x 10^5 cells)	
No mitogen	7778 ± 4959	10179 ± 6601
Con A (1 µg)	97654 ± 58212	118697 ± 53177
Con A (5 µg)	165116 ± 68393	195746 ± 62703
Con A (10 µg)	55920 ± 74316	51333 ± 74816
LPS (1 µg)	51064 ± 33406	83905 ± 42547
LPS (5 µg)	53138 ± 33170	90994 ± 41820
LPS (20 µg)	49571 ± 34632	83618 ± 44002

[a]Values represent the mean cpms ± SD derived from 75 male and female control CD-1 mice used in five 90-day studies. The means for each parameter were significantly different ($p < 0.05$) among the five experiments.

plaque assay[12]. IgM AFC were enumerated 4 and 5 days following intraperitoneal immunization with 4 x 10^8 sRBC. Data are presented as AFC/10^6 spleen cells and also as AFC/spleen. Historical control data for IgM AFC from six 90-day studies are shown in Table 16.

Hemagglutination: Mice were immunized intraperitoneally with 1 x 10^9 sRBC on day 0. On day 7, blood was collected into 3.2% sodium citrate as previously described. A sample of the plasma was removed and heat inactivated at 56°C for 30 min. Serial 1:1 dilutions were made in phosphate buffered saline in round bottom 96-well microtiter plates to a final volume of 0.1 ml. To each well was added 0.1 ml of a 0.5% sRBC solution. The plates were covered and placed in a 37°C humidified incubator.

TABLE 16
Control Values for Humoral Immunity as Measured by Spleen IgM AFC and Hemagglutination in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Male		Female	
	Day 4	Day 5	Day 4	Day 5
Spleen Weight	189 ± 61	177 ± 38	196 ± 44	183 ± 55*
Spleen Cell No. ($\times 10^8$)	1.67 ± 0.59	1.54 ± 0.44	1.68 ± 0.52	1.65 ± 0.52
IgM AFC/ Spleen ($\times 10^{-5}$)	3.15 ± 1.53	1.64 ± 0.94	3.54 ± 1.47	1.92 ± 0.73
IgM AFC/10^6 Spleen Cells	1851 ± 488	1016 ± 401	2114 ± 737	1200 ± 412
Hemagglutination Titer (Log_2)	9.39 ± 0.89		9.63 ± 1.0	

[a]Values represent the mean ± SD derived from 70 female and male CD-1 mice used in six subchronic 90-day studies. Hemagglutination titers were derived from 48 mice used in four subchronic 90-day studies. The assays were carried out as described in Methods. The means for each parameter were significantly different ($p < 0.05$) among the experiments, except where indicated by an asterisk.

Two hr later, the plates were removed and placed on a magnifying mirror to determine at which serum dilution no agglutination occurs. The historical control data from four 90-day studies evaluating hemagglutination are shown in Table 16.

Macrophage Function

Functional Ability of the Reticuloendothelial System (RES): Five ml of freshly drawn sRBC (5×10^9 cells/ml) were radiolabeled with 1 mCi of sodium chromate-51 (New England Nuclear) in a 37°C shaker bath for 30 min. After chromation, the sRBC

were washed with Alsever's solution until the supernatant was virtually radioactivity free. Unlabeled sRBC (5×10^9/ml) were added to the labeled cells until the hematocrit was approximately 12%. The resulting cpms were 200,000/0.1 ml. The sRBC were refrigerated and used the following day. Before use, the cells were washed to remove any free chromium which was release overnight, and resuspended in phosphate buffered saline (PBS).

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 39°C. At 0 time, 0.1 ml of labeled particle/10 g body weight was injected intravenously. Ten µl blood samples were taken from the tip of the tail at 2, 4, 6, 8, 10, and 15 min. At 60 min, mice were sacrificed by decapitation and selected organs collected. The blood samples were put into 1 ml of distilled water and radioassayed. Blood clearance was expressed as the phagocytic index, which was determined by the slope of the clearance curve.

Figure 3 shows the time course of particle uptake from the selected organs. For a routine study, mice were sacrificed at the plateau period (60 min after injection). Liver, spleen, lungs, thymus, and kidneys were removed, weighed, and counted in a gamma counter. Organ distribution was expressed as percent organ uptake and cpms/mg tissue (specific activity). Control values from six 90-day studies are given in Table 17.

Recruitability of PEC: Mice were injected intraperitoneally with 1 ml of 10% Brewer's thioglycolate on day 0. On day 5, the mice were sacrificed by cervical dislocation, and the peritoneal cavity was flushed with 10 ml of minimum essential medium (MEM). The cells were centrifuged at 300 xg for 10 min and then suspended in fresh MEM. A sample was removed and enumerated on a Coulter counter in the presence of a lysing agent to determine the number of PEC recruited.

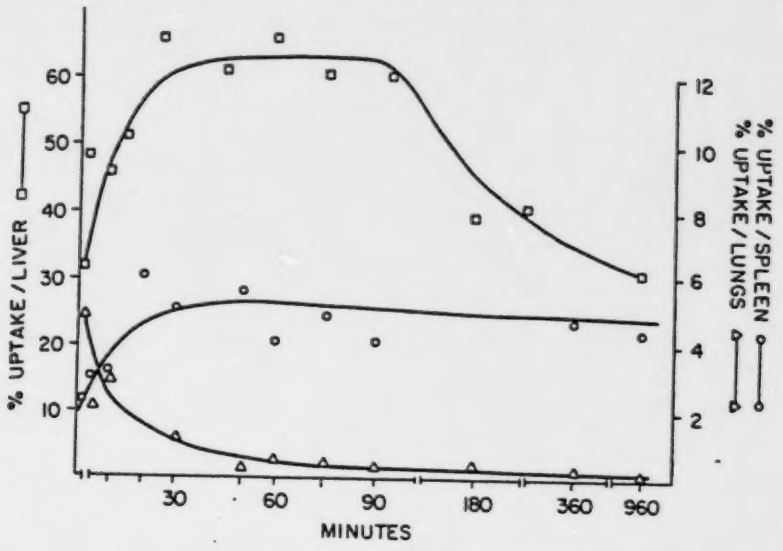

FIG. 3. The time course of particle uptake in liver, spleen, and lung. See Methods for procedure.

TABLE 17
Control Values for the Functional Activity of the RES as Measured by Vascular and Organ Clearance of Cr-51 Labeled sRBC in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Male		Female	
	% Uptake	cpm/mg	% Uptake	cpm/mg
Phagocytic Index	0.10 ± 0.05		0.11 ± 0.07	
Liver	49.6 ± 11.1	217 ± 66	57.6 ± 10.6	247 ± 69
Spleen	8.81 ± 4.08	552 ± 299	10.6 ± 6.2	493 ± 332
Lung	1.31 ± 1.11	43 ± 46	1.27 ± 0.93*	35 ± 28
Thymus	.006 ± .002	4 ± 3*	.010 ± .007	6 ± 6
Kidney	2.29 ± 1.10*	30 ± 17	2.27 ± 0.79	36 ± 13

[a]Values represent the mean ± SD derived from 95 control CD-1 mice used in six 90-day studies. The means for each parameter were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

Phagocytic Activity of PEC: PEC were collected, resuspended, and counted as described above. Cell concentrations were adjusted to 2 x 10^5/ml and 1 ml samples added to each well of a 24-well Costar dish. Plates were incubated for one hr in a humidified CO_2 incubator (37°C). The medium was decanted and the adherent cells were washed with MEM. To each well was added 5 µl of chromated sRBC (~100,000 cpm) prepared as described above, which had been opsonized with mouse IgG. The adhered PEC and ^{51}Cr sRBC were incubated for 10, 20, 30, and 45 min. The plates were then washed once with distilled water and then twice with medium to remove any adhered sRBC. One N NaOH (1 ml) was then added to each well to dissolve the PEC and then the contents of the wells were counted in a gamma counter.

Adherence of PEC: PEC were collected, resuspended, and counted as described previously. Cell concentrations were adjusted to 2 x 10^5/ml and 1 ml samples were placed in each well of a 24-well Costar plate. The plates were incubated for 18-24 hr in a 37°C humidified CO_2 incubator. The cells were then washed extensively, scraped from the plates, and counted on a Coulter counter.

Chemotaxis of PEC: Chemotaxis of PEC was measured in modified blind well Boyden chambers using 13 mm Sartorius membrane filters as described by Laskin et al.[13] Varying numbers of cells (1 to 2 x 10^6 cells/ml) were placed in the upper portion of the chamber, with vehicle or chemoattractant (endotoxin activated mouse serum) in the lower portion. After incubation at 37°C for 4 hr, the filters were removed, rinsed in methanol, and fixed in formalin until stained. They were then placed on glass slides, stained with hematoxylin, rinsed, dipped in 0.1% NH_4OH for 1 min, and rinsed in distilled water. After processing through 95% ethanol, absolute ethanol, and isopropanol, the filters were cleared in xylene, mounted on glass slides, and

TABLE 18
Control Values for PEC Parameters in Male and Female CD-1 Mice Used in 90-Day Studies[a]

Parameter	Male	Female
Recruitable PEC ($\times 10^{-7}$)	1.0 ± .11	2.0 ± .17
Adherent PEC ($\times 10^{-5}$)	2.6 ± .08	2.6 ± .06
Chemotaxis (cells/field)		
No Chemoattractant	22.6 ± 6	26.0 ± 6
EAMS 1:10	167 ± 27	68 ± 10
Phagocytosis of Ab-^{51}Cr sRBC (cpm)		
10 minutes	596 ± 99	354 ± 58
20 minutes	1086 ± 182	449 ± 66
30 minutes	1420 ± 216	604 ± 83
45 minutes	1693 ± 233	745 ± 112

[a]Values represent the mean ± SD of 32 male and female CD-1 mice, derived from three 90-day studies. EAMS = endotoxin activated mouse serum, Ab-^{51}Cr sRBC = ^{51}Cr labeled sRBC opsonized with mouse IgG.

dried. Using a Nikon microscope interfaced with an Artec Model 980 Image Analyzer, 20 microgrid fields were counted.

Historical control values for PEC parameters are given in Table 18.

Statistical Evaluation

If a one way analysis of variance of the means showed study to study differences, a Duncan's Multiple Range Test was performed[14]. Values which differ from study to study at $p < 0.05$ were considered statistically significant. Each of the values represents the mean ± SE.

DISCUSSION

We have chosen to approach the problem of evaluating the effects of chemicals on the lymphoreticular system from a toxi-

cological viewpoint. Acute, 14- and 90-day studies were utilized for each chemical. The acute study was used to aid in selecting doses for subsequent studies and suggesting possible toxicological target sites. In the 90-day studies, both male and female mice were used. The outbred CD-1 mouse was our animal of choice and, as an outbred strain, the increased animal-to-animal variation was acceptable. Due to the random genetic background of the mice, many of the parameters monitored did not fall into a normally distributed response. Most notable is the variation in the number of AFC responding to sRBC in control animals. There appear to be at least three distinct populations of animals: low responders, medium responders, and high responders. In an attempt to circumvent this problem, a large number of mice was used for each treatment dose and for each parameter evaluated.

The effects of a chemical on the immune response were not studied independently, but along with a multitude of standard toxicological parameters. This results in a more complete overview of the chemical's effect. In evaluating the effects of a compound, specifically on the immune system, not just one, but several assays were used in assessing humoral immunity, cell-mediated immunity, and macrophage function.

It has been our experience that historical controls can only be used as a guide. As repeatedly pointed out in this paper, experiments using the same strain of mice, obtained from the same supplier, and handled in an identical manner by the same people results in control values which will differ significantly from experiment to experiment at the $p < 0.05$ level as determined by a one way analysis of variance. Thus, historical controls are only beneficial in establishing a data base. When comparing data for safety assessment or evaluation of drug effects, one must compare the treatment groups to the appropriate concurrent controls used in the actual study.

Since the assays outlined here can be used to evaluate the status of the immune system in a manner which is quantifiable and reproducible, the assessment of the immunotoxicity of chemicals can be easily incorporated into routine toxicity testing. These methods have been used in this laboratory to evaluate the toxicity and immunotoxicity of some halogenated hydrocarbons found to be drinking water contaminants[15,16,17]. The following four reports use these same approaches to assess the general toxicity and immunotoxicity of two other drinking water contaminants, trans-1,2-dichloroethylene (DCE) and 1,1,2-trichloroethane (TCE).

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